ARTICLE
Journal of Cellular Biochemistry 106:937–955 (2009)

Journal of Cellular Biochemistry

Outcome of Treatment of Human HeLa Cervical Cancer Cells With Roscovitine Strongly Depends on the Dosage and Cell Cycle Status Prior to the Treatment

Józefa Węsierska-Gądek,* Andreea Borza, Eva Walzi, Vladimir Krystof, Margarita Maurer, Oxana Komina, and Stefanie Wandl

Cell Cycle Regulation Group, Department of Medicine I, Div. Institute of Cancer Research, Medical University of Vienna, Vienna, Austria

ABSTRACT

Exposure of asynchronously growing human HeLa cervical carcinoma cells to roscovitine (ROSC), a selective cyclin-dependent kinases (CDKs) inhibitor, arrests their progression at the transition between G_2/M and/or induces apoptosis. The outcome depends on the ROSC concentration. At higher dose ROSC represses HPV-encoded E7 oncoprotein and initiates caspase-dependent apoptosis. Inhibition of the site-specific phosphorylation of survivin and Bad, occurring at high-dose ROSC treatment, precedes the onset of apoptosis and seems to be a prerequisite for cell death. Considering the fact that in HeLa cells the G_1/S restriction checkpoint is abolished by E7, we addressed the question whether the inhibition of CDKs by pharmacological inhibitors in synchronized cells would be able to block the cell-cycle in G_1 phase. For this purpose, we attempted to synchronize cells by serum withdrawal or by blocking of the mitotic apparatus using nocodazole. Unlike human MCF-7 cells, HeLa cells do not undergo G_1 block after serum starvation, but respond with a slight increase of the ratio of G_1 population. Exposure of G_1-enriched HeLa cells to ROSC after re-feeding does not block their cell-cycle progression at G_1-phase, but increases the ratio of S- and G_2-phase, thereby mimicking the effect on asynchronously growing cells. A quite different impact is observed after treatment of HeLa cells released from mitotic block. ROSC prevents their cell cycle progression and cells transiently accumulate in G_1-phase. These results show that inhibition of CDKs by ROSC in cells lacking the G_1/S restriction checkpoint has different outcomes depending on the cell-cycle status prior to the onset of treatment. J. Cell. Biochem. 106: 937–955, 2009.

KEY WORDS: APOPTOSIS; CELL CYCLE ARREST; CYCLIN-DEPENDENT KINASES; INHIBITORS OF CYCLIN-DEPENDENT KINASES; ROSCOVITINE

Altered expression of cell cycle regulatory genes contributes to uncontrolled proliferation of malignant cells. Cyclin-dependent kinases (CDKs), the key enzymes governing progression through the cell cycle, are subject of frequent deregulation through genetic or epigenetic mechanisms providing the cells selective growth advantage [Malumbres and Barbacid, 2005, 2007]. These enzymes are composed of a catalytic kinase subunit and a regulatory cyclin subunit that is essential for activation of CDKs. Cyclins themselves are expressed only during particular cell cycle periods, allowing activation of individual CDKs at the right time. Besides, CDKs are regulated by stimulating phosphorylation of the activation segment catalyzed by CDK-activating kinase (CAK), by inhibitory phosphorylation within the ATP-binding site induced by Wee/Myt kinases, and finally by their interactions with small protein inhibitors (Cip/Kip or INK4) [Malumbres and Barbacid, 2005; Besson et al., 2008]. All these proteins cooperate in a balanced network in normal cells, whereas abnormal CDK regulation is a hallmark of cancer cells. CDKs are mutated in cancers only seldom, with the rare exception of a point mutation in CDK4, resulting in the loss of INK4 binding, or more often by gene amplifications and overexpression of both CDK4 and CDK6 [Malumbres and Barbacid, 2005]. However, a significant number of cancers bear changes in

Abbreviations used: CAK, cyclin-dependent kinase-activating kinase; CDK, cyclin-dependent kinase; Cip/Kip, CDK-interacting protein/kinase-inhibiting protein; HPV, human papilloma virus; INK4, inhibitor of CDK4; NOC, nocodazole; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene difluoride; pRb, retinoblastoma protein; ROSC, roscovitine; Waf1, wild-type p53-activated fragment 1.

Grant sponsor: FWF; Grant number: P-19894-B11.

*Correspondence to: Józefa Węsierska-Gądek, Cell Cycle Regulation Group, Div. Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria.
E-mail: jozefa.gadek-wesierski@meduniwien.ac.at

Received 12 August 2008; Accepted 23 December 2008 • DOI 10.1002/jcb.22074 • 2009 Wiley-Liss, Inc.
Published online 29 January 2009 in Wiley InterScience (www.interscience.wiley.com).

genes encoding CDK regulatory partners, including cyclins E and D, Cip/Kip and INK4 inhibitors, and their substrates (mainly retinoblastoma protein, pRb) [Malumbres and Barbacid, 2007; Besson et al., 2008]. This permits escape from senescence during malignant transformation and allows cancer cells to proliferate with increased CDK activity and accumulate further mutations.

On the basis of deregulated activity of CDKs detected in cancers, inhibitors targeting specifically these kinases have been developed as anticancer therapeutics. A growing number of small molecule inhibitors are now in preclinical testing or even in clinical trials [Fischer and Lane, 2000; Dai and Grant, 2003; Malumbres et al., 2008]. The most success so far has been obtained with ATP-mimicking competitive inhibitors of CDK. They are usually classified according to their specificities as pan-specific (do not discriminate among different CDKs) and oligo-specific (preferentially inhibit only some CDKs). PD-0332991, which has been developed to preferentially target CDK4/6, is a typical example of the latter group [Fry et al., 2004]. However, the majority of developed drugs, including flavopiridol, roscovitine (ROSC), or SNS-032, hit simultaneously multiple CDKs. These oligo-specific compounds potently arrest the cell cycle in transformed cells, due to inhibition of cell cycle regulating CDK1, CDK2, and/or CDK4, and induce apoptosis as well, apparently as a result of transcriptional perturbations caused by CDK7 and CDK9 inactivation [Shapiro, 2006].

Interestingly, several pharmacological CDK inhibitors also potently block transcription and replication of some viruses, such as HIV, HCMV, VZV, HSVs, and EBV, that rely on the activity of endogenous CDKs in host animal cells [Schang et al., 2005]. Some viruses can inactivate pRb through direct binding of viral proteins and the consequent abrogating of the G_1 restriction point, the others are able to constitutively activate cellular CDKs by virally encoded cyclin proteins [Helt and Galloway, 2003], or even encode kinases able to substitute CDKs in their ability to phosphorylate pRb [Hume et al., 2008]. It is assumed that the antiviral activity of CDK inhibitors is based on interference with viral transcription. Importantly, as some human cancers are induced by oncogenic viruses, like Kaposi's sarcoma or HPV-induced cervical carcinoma, therapy based on CDK inhibitors would not only stop the proliferation of transformed cells, but also advantageously limit the life-cycle of the transforming viruses, both dependent on cellular CDKs.

Human papilloma viruses (HPV) are small double-stranded DNA viruses infecting squamous epithelia, inducing proliferative lesions within infected tissues that are prerequisite for the development of cancer. The high risk HPV-encoded proteins E6 and E7, substantially contributing to transforming capability, play a major role in the deregulation of the cell cycle control. Protein E6 binds p53 and directs it to ubiquitin-mediated degradation, further promoting genetic instability in infected cells [Scheffner et al., 1990]. E7 not only induces aberrant S-phase entry through the inactivation of pRb tumor suppressor and related pocket-proteins, but also increases expression of E2F-responsive cell cycle regulators such as the cdc25a tyrosine phosphatase that further contribute to G_1/S deregulation [Nguyen et al., 2002]. Moreover, E7 prevents $p21^{Waf1}$ both from inhibiting activity of CDK2/cyclin E and DNA replication and thus disrupts normal cell cycle control by increased CDK2 activity and enhanced expression of E2F-responsive genes [Funk et al., 1997]. As shown in mouse models, inactivation of pRb by E7 is not sufficient to overcome G_1 restriction point; also other E2F regulators are important targets of E7 that play critical roles in cervical carcinogenesis [Balsitis et al., 2006]. For example, recent experiments demonstrated that E7 can associate with and inactivate the transcriptional repression activity of E2F-6, thereby extending S-phase competence of HPV infected cells [McLaughlin-Drubin et al., 2008]. Down-regulation of both viral oncoproteins increases the efficacy of the therapy [Wesierska-Gadek et al., 2002].

In the light of the facts mentioned above, the question appeared whether the inhibition of cellular CDKs by pharmacological inhibitors would be able to reduce the proliferation rate of cancer cells infected with high risk HPV and to restore the proper cell cycle control as well as to optionally trigger them to undergo apoptosis. For this purpose we decided to apply ROSC, a relatively selective CDK inhibitor targeting not only kinases regulating cell cycle progression but also CDK7, the dual acting kinase that is also implicated in the regulation of transcription [Fisher, 2005]. Considering the fact that cellular RNA polymerase II is required for transcription of HPV-encoded proteins and that CDK7 complexed with cyclin H is a constituent of the basal transcription factor TFIIF, which phosphorylates the serine residues within the heptapeptide repeat of the carboxy terminal domain (CTD) of RNA polymerase II, one might expect that ROSC would not only affect the functionality of the intrinsic cellular cell cycle regulators but would also prevent transcription of genes encoded by the infecting pathogen. We chose human HeLa cervical carcinoma cells, the most frequently investigated cell line derived from HPV-positive cervical carcinoma, as an experimental model. The action of ROSC was previously studied in detail in asynchronously growing [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a, 2006, 2007c] and partially synchronized human MCF-7 breast cancer cells [[David-Pfeuty, 1999] as well as in normal healthy cells [Alessi et al., 1998; Wesierska-Gadek et al., 2007a]. The susceptibility of asynchronously growing MCF-7 cells and cells released from a partial mitotic block substantially differed. Exposure of MCF-7 cells released from a partial mitotic block to ROSC slowed their G_1 to S progression [David-Pfeuty, 1999]. In contrast, ROSC arrested asynchronously growing MCF-7 cells in the G_2 phase of the cell cycle, and after longer exposure induced caspase-3 independent apoptosis. ROSC-induced cell death was mediated by wt p53. ROSC strongly enhanced the cellular expression of p53 protein and markedly extended its half-life [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. Interestingly, ROSC-induced phosphorylation of p53 protein at serine 46 resulted in an up-regulation of p53AIP1 protein, its specific downstream target that after de novo synthesis and translocation into the mitochondria [Wesierska-Gadek et al., 2005a] initiated apoptosis in MCF-7 cells which lack caspase-3. Unlike in asynchronously growing MCF-7 cells, in those released from a partial mitotic block, ROSC slowed the transition of cells from G_1 to S phases [David-Pfeuty, 1999]. Surprisingly, reconstitution of MCF-7 cells with human caspase-3 did not sensitize them to the action of ROSC [Wesierska-Gadek et al., 2005a]. ROSC failed to induce P-Ser46 p53 protein and its downstream target in asynchronously growing, caspase-3 proficient MCF-7 cells [Wesierska-Gadek et al., 2005a], thereby indicating that

the outcomes of the inhibition of cellular CDKs strongly depends on cell types, their intrinsic features and cell cycle status prior to the onset of therapy. This observation was additionally substantiated by the fact that phenol red, a weakly estrogenic component of culture medium, affects the susceptibility of exponentially growing MCF-7 cells, that are known to express ER-α, to the action of ROSC [Wesierska-Gadek et al., 2006].

In the light of the above discussed cognitions it is incontrovertible that the outcomes of action of CDK inhibitors cannot be exactly predicted in different tumor cell lines, especially in those differing in the status of cell cycle and apoptosis regulators.

In this work we studied the effect of the CDK inhibitor ROSC on normal MRC-5 human fibroblasts and two human cervical carcinoma cell lines (HTB-31 and HeLa cells). HeLa cells are positive for HPV-18, while in HTB-31 no viral infection was detected. Both cancer cell lines differ in the p53 status. We determined the anti-proliferative and pro-apoptotic effect of ROSC on exponentially growing cells. Inhibition of CDKs most strongly affects the proliferation of HeLa cells. The marked reduction of the number of viable cells upon exposure of HeLa cells to ROSC is attributable to induction of cell cycle block at G_2/M and/or apoptosis. The outcome depends on the ROSC concentration. At higher dose ROSC eliminates HeLa cells by apoptosis. Moreover, ROSC represses the HPV-encoded oncoproteins. Unlike HeLa, HTB-31 cancer cells are markedly less susceptible to the tested CDK inhibitors. The ROSC-induced accumulation of the S- and G_2/M-phase population is not accompanied by apoptosis.

We also addressed the question whether the inhibition of CDKs in synchronized cells would be able to block the cell cycle in G_1 phase. For this purpose, we attempted to synchronize cells by serum withdrawal or by blocking the mitotic apparatus using nocodazole. Our results show that inhibition of CDKs in cells lacking the G_1/S checkpoint has different outcomes depending on the cell cycle status prior to the onset of treatment; ROSC does not inhibit HeLa cells in G_1-phase, but is solely able to maintain the early G_1-phase cell cycle arrest of cells released from the nocodazole-induced mitotic block. The results clearly show that ROSC effectively restricts growth of HeLa cells and the outcome is dose-dependent. It became evident that a higher ROSC concentration is necessary to promote apoptosis through abrogation of the activity of its inhibitors.

MATERIALS AND METHODS

CELLS

The human cervical carcinoma cell lines $HeLaS_3$, HTB-31 (C-33A), and normal MRC-5 human fibroblasts obtained from American Type Culture Collection (ATCC), were cultured in RPMI medium supplemented with 10% foetal calf serum (FCS). Cells were grown up to 60–70% confluence and then treated with indicated drugs.

DRUGS

Roscovitine (ROSC) was prepared according to the published procedure [Havlicek et al., 1997] and prepared as 50 mM stock solution in DMSO. Aliquots of the stock solution were stored until use at −20°C. Nocodazole (NOC) from Sigma–Aldrich, Inc. (St. Louis, MO) at a final concentration of 0.05 μg/ml was used to induce mitotic block.

ANTIBODIES

The following specific antibodies were used to detect the relevant proteins: monoclonal anti-p53 antibody DO-1 (a kind gift from Dr. B. Vojtesek, Masaryk Memorial Cancer Institute, Czech Republic), the polyclonal anti-phospho-Thr161 CDK1, anti-phospho-Thr14/Tyr15 CDK1, anti-phospho-Thr160 CDK2, anti-phospho-Ser216 CDC25C, anti-phospho-Ser780 pRb, anti-phospho-Ser807/81 pRb, anti-phospho-Ser139-H2A.X, anti-phospho-Ser112 Bad, and corresponding antibodies against the total antigen (all from New England Biolabs, Beverly, MA), polyclonal anti-phospho-Ser164/Thr170 CDK7 and anti-phospho-Ser199 NPM (BioLegend, San Diego, CA), anti-caspase-3 (DAKO AS, Glostrup, Denmark), monoclonal anti-CDK2 (Ab-4) antibodies (Lab Vision Co., Fremont, CA), polyclonal-anti-phospho-Thr34 survivin, monoclonal anti-PCNA (clone PC-10), anti-pRb (IF-8), anti-cyclin A, anti-NPM (all from Santa Cruz Biotechnology, CA), anti-CDK7 (clone MO-1.1, Sigma–Aldrich, Inc.), anti-actin (clone C4, ICN Biochemicals, Aurora, OH), anti-E7 oncoprotein were from Abcam Ltd (Cambridge, England). Appropriate secondary antibodies linked to horseradish peroxidase (HRP) were from R&D Systems (Minneapolis, MN).

DETERMINATION OF THE NUMBER OF VIABLE CELLS

Proliferation of human cervical carcinoma cell lines $HeLaS_3$, HTB-31 (C-33A), and normal MRC-5 human fibroblasts and their sensitivity to increasing concentrations of ROSC was determined by the CellTiter-GloTM Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). As described recently in more detail [Wesierska-Gadek et al., 2005b], the CellTiter-Glo™ Luminescent Cell Viability Assay, generating luminescent signal, is based on quantification of the cellular ATP levels. Tests were performed at least in quadruplicates. Luminescence was measured in the Wallac 1420 Victor, a microplate luminescence reader. Each point represents the mean ± SD (bars) of replicates from at least three experiments.

DETERMINATION OF CASPASE-3/7 ACTIVITY

The activity of both caspases was determined using the APO-ONE Homogenous Caspase-3/7 Assay (Promega Corporation) which uses the caspase-3/7 substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R100) as described previously [Wesierska-Gadek et al., 2005b]. Briefly, $HeLaS_3$ cells were plated in 96-well microtiter plates. One day after plating the cells were exposed for 24 h to increasing drug concentrations. Thereafter, the culture supernatant was transferred into another microtiter plate to separately determine the caspase activity in cells and in culture medium. Then an equal volume of caspase substrate was added and samples were incubated at 37°C for different periods of time to assess the best signal-to-background ratio. The fluorescence was measured at 485 nm. Luminescence and fluorescence were measured in the Wallac 1420 Victor, a microplate luminescence reader. Each point represents the mean ± SD (bars) of replicates from at least three experiments.

MEASUREMENT OF THE DNA CONTENT OF SINGLE CELLS BY FLOW CYTOMETRY

Measurement of the DNA-content was performed by flow cytometric analysis based on a slightly modified method [Wesierska-Gadek and Schmid, 2000] described previously by Vindelov et al. [1983]. The cells were detached from substratum by limited trypsinization, then all cells were harvested by centrifugation and washed in PBS. Aliquots of 10^6 cells were used for further analysis. Cells were stained with propidium iodide as described previously and then the fluorescence was measured using the Becton Dickinson FACScan after at least 2 h incubation at 4°C in the dark.

IMMUNOBLOTTING

Total cellular proteins dissolved in SDS sample buffer were separated on SDS slab gels, transferred electrophoretically onto PVDF membrane (PVDF) (Amersham Biosciences), and immunoblotted as previously described [Wojciechowski et al., 2003]. Equal protein loading was confirmed by Ponceau S staining. To determine the phosphorylation status of selected proteins, antibodies recognizing site-specifically phosphorylated proteins were diluted to a final concentration of 1:1,000 in 1% BSA in Tris-saline-Tween-20 buffer. In some cases, blots were used for sequential incubations.

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software and significance levels were evaluated using Bonferroni's Multiple Comparison Test.

RESULTS

ROSC MORE STRONGLY INHIBITS THE PROLIFERATION OF HUMAN HeLa CERVICAL CANCER CELLS THAN THAT OF HTB-31 CELLS

To determine the effect of ROSC on the proliferation of exponentially growing human HeLa cells, the cells were continuously exposed to the drug for 12, 18, or 24 h, respectively. Then the cell number was determined using CellTiter-Glo viability assay immediately upon termination of the treatment, or alternatively the medium was changed, and then cells were post-incubated in a drug-free medium for a further 2 days and thereafter the assay was performed (Fig. 1A). According to the statistical analysis, 40 μM ROSC very significantly reduced the number of living cells already after 12 h treatment. The effect was even stronger after longer incubation periods (18 and 24 h) and persisted upon medium change. Reduction of living cells following treatment with lower doses of ROSC (i.e., 10 and 20 μM) was not statistically significant over the 24 h period. However, when cells incubated with 20 μM ROSC for 24 h were transferred to the drug-free medium, the number of living cells was significantly reduced after the further 48 h cultivation.

In contrast, human HTB-31 cancer cells are less susceptible to the action of CDK inhibitors. Approximately a fourfold higher dose of ROSC is required to reduce the number of viable cells by 50% within 24 h (Fig. 1B).

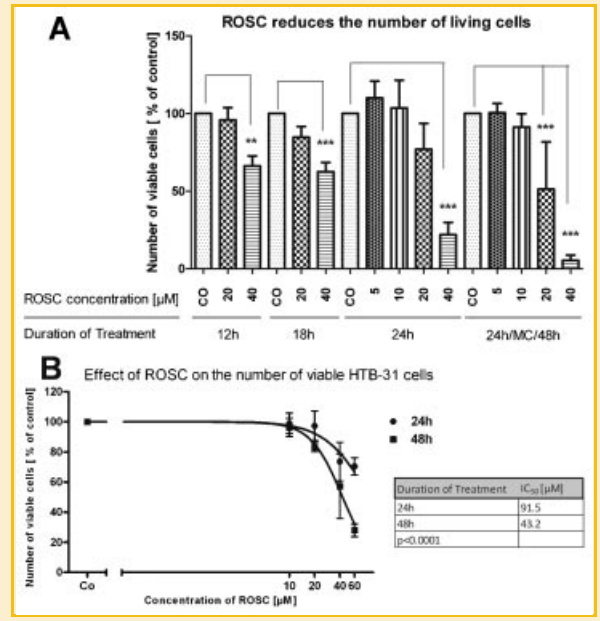

Fig. 1. Reduction of the number of viable human HeLa and HTB-31 cervical cancer cells after treatment with ROSC. A: Exponentially growing HeLa cells plated in 96-well microtiter plates were treated for 12, 18, and 24 h, respectively, with indicated concentrations of ROSC. The number of viable cells was determined directly after the treatment, or eventually after medium change (MC) and post-incubation for 48 h in a drug-free medium. The graph represents mean values from three independent experiments, each performed at least in quadruplicates. B: Exponentially growing HTB-31 cells plated in 96-well microtiter plates were treated for 24 and 48 h with ROSC. The differences between the number of control and treated cells are statistically highly significant (**; $0.001 < P < 0.01$) or very highly significant (***$P < 0.001$) according to the Bonferroni's comparison.

As expected, normal human fibroblasts (MRC-5) were only negligibly affected by the selective CDK inhibitor. The IC_{50} values are shown in Table I.

ROSC INHIBITS THE CELL CYCLE OF ASYNCHRONOUSLY GROWING HeLa CELLS AT THE G_2/M TRANSITION

The next experimental series were performed to find out how ROSC modulates the cell cycle progression and whether it is also able to induce apoptosis in HeLa cells. The DNA concentration in single cells was measured by flow cytometry. The population of hypoploid cells representing cells undergoing apoptotic changes was classified as a sub-G_1 population. HeLa cells were exposed to two concentrations of ROSC. As shown in Figure 2, ROSC at a final concentration of 20 μM increased the frequency of G_2/M population and concomitantly

TABLE I. Treatment With ROSC for 24 h

Cell line	IC_{50} (μM)
HeLa	28.1
HTB-31	91.5
MRC-5	Not achieved

diminished that of G_1-phase, with transient accumulation of S-phase after 18 h treatment. Two times higher concentration of ROSC increased the frequency of G_2/M population even more strongly at the same time point, but simultaneously, the population of hypoploid cells (30%) appeared after incubation for 18 and 24 h.

Concurrently, whole cell lysates obtained from HeLa cells treated with ROSC were analyzed by immunoblotting to monitor changes in main cell cycle protein regulators (Fig. 3A,B). According to the predictions, phosphorylation of CDK2 at Thr160 was diminished in ROSC-treated cells indicating decreased activity of this kinase already after 12 h. The kinase responsible for activation of CDK2 by phosphorylating its Thr160 is CDK7, whose reduced phosphorylation at Ser164/Thr170 suggests one possible mechanism by which ROSC leads to inactivation of CDKs in cells. As expected,

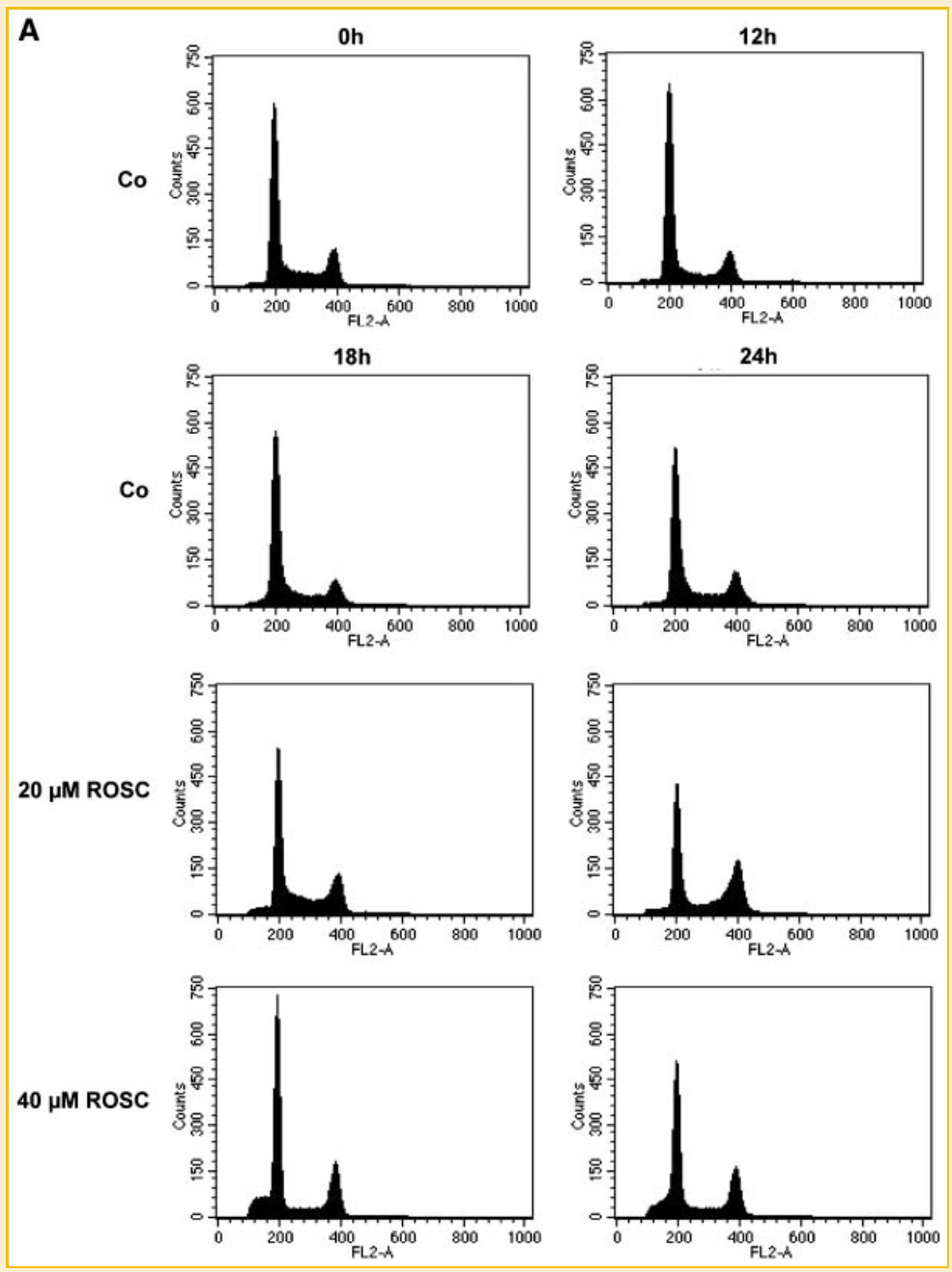

Fig. 2. Effect of ROSC on the cell cycle progression of asynchronously growing HeLa cells. Exponentially growing HeLa cells were treated with either 20 or 40 μM ROSC for the indicated periods of time. Control and drug-treated cells were harvested by trypsinization and single cell suspension was used for propidium iodide staining. DNA content in single cells was measured using the Becton Dickinson FACScan flow cytometer. DNA histograms depicting a representative experiment performed in duplicate were prepared using the CellQuest evaluation program (A) and diagrams showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G_1 cell population were constructed (B). The distribution was determined using ModFIT cell cycle analysis software. The values represent a mean of two replicates.

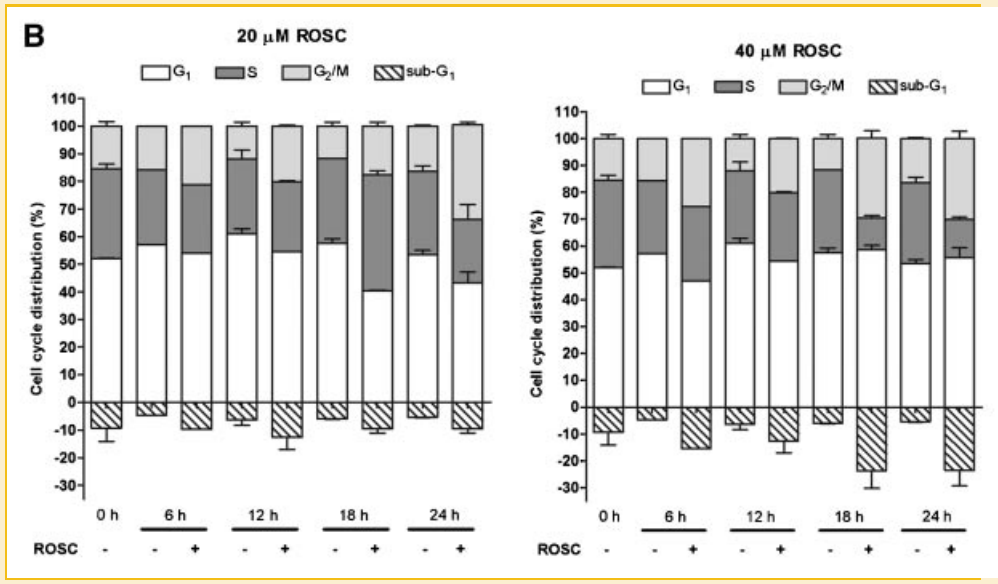

Fig. 2. (*Continued*)

ROSC prevented phosphorylation of pRb at Ser780 and Ser807/811, which correlates with decreased proliferation and observed reduction of G_1-phase in treated cells. However, pRb is phosphorylated by several CDKs, and one cannot discriminate which kinase is responsible for this effect. Therefore, we also analyzed phosphorylation of another CDK2-specific substrate—nucleophosmin (NPM) (Fig. 3B). A reduced signal from its phosphorylation at Thr199 is another evidence for inactivated CDK2. Surprisingly, after exposure of cells to 40 μM ROSC for 24 h the site-specific phosphorylation of NPM increased. It remains to prove whether this event might be linked in any way to the ongoing apoptosis.

REPRESSION OF THE CELLULAR LEVELS OF HPV-ENCODED ONCOPROTEINS UPON ROSC TREATMENT

One might expect that inactivation of CDK7 and of cellular RNA polymerase II would shut down the expression of HPV-encoded proteins. Indeed, ROSC at high dosage reduced the levels of E6 (not shown) and E7 oncoproteins (Fig. 3C). The decrease of E7 protein was observed after treatment with ROSC for 18 h.

HIGHER DOSES OF ROSC INDUCE CASPASE-DEPENDENT APOPTOSIS IN HeLa CELLS

The exposure of HeLa cells to 40 μM ROSC for 24 h resulted in a marked reduction of the number of viable cells. This effect seems to be attributable not only to the cell cycle arrest but additionally to the increased accumulation of a sub-G_1 cell population as detected by flow cytometry (Fig. 2), which is an indication of apoptosis. To substantiate this assumption, activity of caspase-3/7 in control and ROSC-treated cells was determined. The activity of cellular caspase-3/7 increased fourfold in HeLa cells treated with 40 μM ROSC after 12 h or even more than eightfold after 24 h (Fig. 4A). Importantly, 20 μM ROSC did not enhance activity of caspase-3/7 in HeLa cells even after 24 h. However, the long term effect of ROSC became evident after medium change and post-incubation of ROSC-treated cells in a drug-free medium for a further 48 h. Activated effector caspases were released into the culture medium. An approximately hundredfold increase of caspase-3/7 activity in culture medium was detected with both doses of ROSC (Fig. 4B).

ROSC-MEDIATED ABROGATION OF PHOSPHORYLATION OF SURVIVIN AND OF BAD PRECEDES THE ONSET OF APOPTOSIS

To identify the mechanism by which inhibition of CDKs may contribute to the initiation of apoptosis, we examined the phosphorylation status of some factors regulating apoptosis. Interestingly, ROSC at the higher dose abolished site-specific phosphorylation of two proteins involved in the regulation of apoptosis: namely of survivin and of Bad (Fig. 5). The decrease of survivin phosphorylation starting after 18 h of exposure to 40 μM but not to 20 μM ROSC became even more evident after further 6 h. Beginning at 12 h treatment ROSC also clearly reduced and thereafter abolished phosphorylation of Bad at Ser112 rendering it disposed to heterodimerize with Bcl-2 proteins. ROSC-mediated decrease of site-specific phosphorylation of Bad did not affect its total level. Interestingly, this was accompanied by down-regulation of the tau form of 14-3-3 protein (Fig. 5). Considering the fact that after treatment with ROSC for 24 h CDK1 is inhibited by modification at Thr14/Tyr15 and (Fig. 3A) and CDC25C phosphatase is inactivated by phosphorylation at Ser216 (Fig. 5), one might speculate that coinciding abrogation of phosphorylation of surviving and Bad trigger apoptosis in G_2/M arrested cells.

The ROSC-mediated abrogation of the phosphorylation of both proteins seems to be a prerequisite for initiation of apoptosis in HeLa cells. Interestingly, exposure of more confluent HeLa cells to high ROSC dose failed to enhance the apoptosis and resulted in solely an accumulation of G_2/M arrested cells (results not shown). Remarkably, in this experimental series the phosphorylation of survivin and Bad was not abolished upon ROSC treatment even at the higher dose

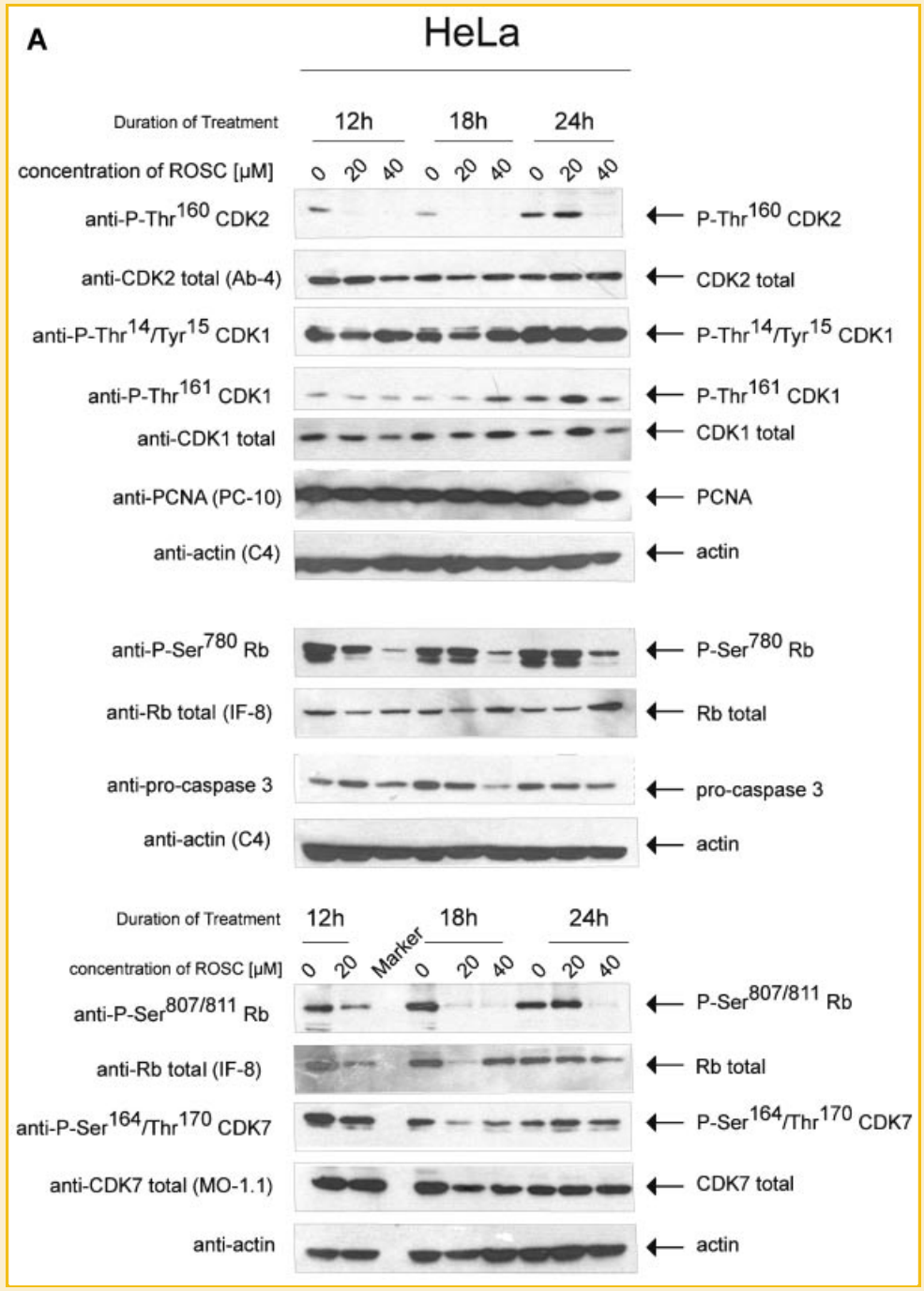

Fig. 3. Cell cycle regulators in asynchronously growing HeLa cells. Untreated and ROSC-treated HeLa cells were harvested and lysed. Whole cell lysates (WCLs) were loaded on 10% or 12% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

(results not shown), thereby indicating that their dephosphorylation is essential for initiation of cell death.

HTB-31 CELLS ACCUMULATE IN G2/M BUT DO NOT DIE AFTER EXPOSURE TO ROSC

The exposure of HTB-31 cells to ROSC inhibits their cell cycle progression. As depicted in Figure 6A,B, HTB-31 cells accumulate in the S- and G_2/M-phase. The frequency of the S-phase population of HTB-31 cells was differentially affected by both ROSC doses (Fig. 6B). At higher dosage percentage of S-phase cells was clearly diminished. After treatment with ROSC at a final concentration of 20 μM frequency of G_1 cell population was clearly reduced. However, no substantial increase of the frequency of hypoploid cells was recorded (Fig. 6A,B).

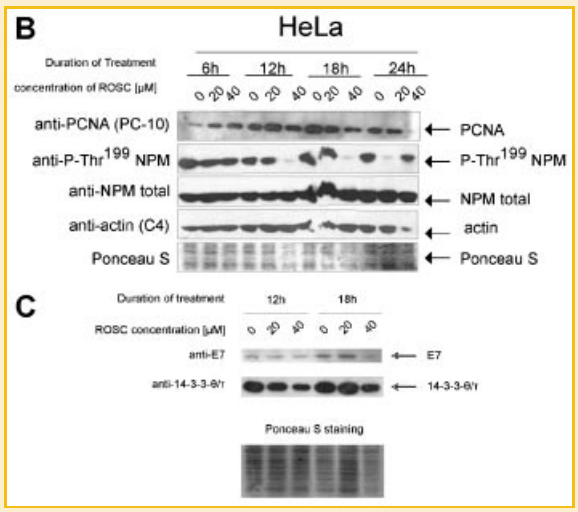

Fig. 3. (*Continued*)

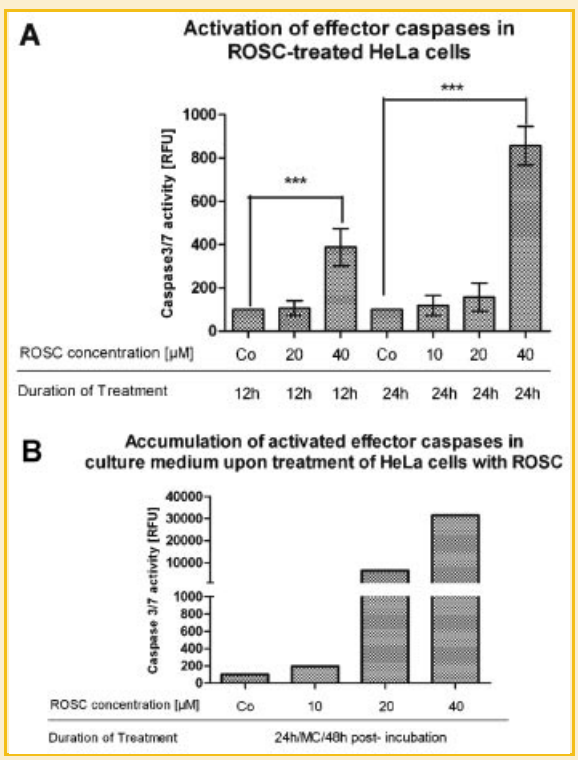

Fig. 4. Activation of caspase-3/7 in ROSC-treated HeLa cells. Exponentially growing HeLa cells were treated in a multiwell plate for either 12 or 24 h with ROSC at indicated concentrations. The activity of cellular caspase-3/7 (A) or caspase-3/7 released to the culture medium (B) was determined in quadruplicates using the APO-One Assay. The caspase-3/7 activity [relative fluorescence units (RFU)] ± SD was normalized to the number of viable cells that were determined by CellTiterGlo Assay. The differences between the caspase-3/7 activity in control and treated cells are statistically very highly significant ($^{***}P < 0.001$) according to the Bonferroni's comparison.

ROSC DOES NOT ACTIVATE EFFECTOR CASPASES IN HTB-31 CELLS

Analysis of WCLs by immunoblotting revealed that levels of procaspase-3 in HTB-31 cells remain unchanged after ROSC treatment for 12 and 24 h (Fig. 6C) indicating that ROSC fails to activate caspase-3. This observation was additionally confirmed by determination of caspase-3/7 activity using the APO-One assay (not shown). Moreover, unlike in HeLa cells, in HTB-31 cells ROSC did not abolish the phosphorylation of survivin and of Bad. However, it is apparent that HTB-31 express lower levels of Bad protein (Fig. 6C) as compared to HeLa cells and other cancer cell lines. The lack of the activation of pro-apoptotic protein and effector caspases correlate with the results of measurement of DNA concentration in single HTB-31 cells performed by flow cytometry (Fig. 6A,B).

EFFECT OF SERUM WITHDRAWAL ON THE CELL CYCLE PROGRESSION OF HeLa CELLS

In the next experiment, the effect of serum withdrawal on the cell cycle progression in HeLa cells was examined. Unlike other cancer cell lines (e.g., MCF-7, used as a positive control), HeLa cells did not undergo G_1 block after serum deprivation when kept in a serum-free medium for 24 or 48 h (Fig. 7A,B). Surprisingly, serum starvation did not induce apoptosis in HeLa cells thereby confirming that the cells are able to survive and proliferate in the absence of growth factors.

Analysis of whole cell lysates obtained from serum-starved HeLa cells by immunoblotting revealed changes in major cell cycle regulators (Fig. 8). During starvation, a slow decrease of the activating phosphorylation of CDK2 and of the total protein level was observed (after 24 h) that was, however, recovered after serum supplementation. Interestingly, simultaneous increase in activating phosphorylation of CDK7 at Ser164/Thr170 was observed in starved cells, followed by its decrease in ROSC-treated cells. The level of site-specific phosphorylation of NPM, which was very low shortly after plating of HeLa cells, strongly increased after their cultivation for further 14 h (Fig. 8). Remarkably, it decreased after withdrawal of serum from the culture medium but not until 20 h and disappeared after serum supplementation (Fig. 8). The total level of nucleophosmin was less affected by these conditions

EFFECT OF ROSC ON HeLa CELLS RELEASED FROM SERUM STARVATION

Serum-starved HeLa cells were not able to arrest in G_1-phase even when sequentially treated with ROSC. A short ROSC treatment (for 12 h) of HeLa cells after re-feeding with serum for 4 h had no effect on the distribution of cells in the cell cycle phases. However, exposure of HeLa cells, serum starved for 24 h and then serum re-fed, to 40 μM ROSC for 24 h markedly reduced the population of G_1 cells and concomitantly led to accumulation of S- and G_2/M-phase cells (Fig. 7C). The distribution of HeLa cells in distinct cell cycle phases upon ROSC is reflected by the status of the main cell cycle regulators (Fig. 9). After a short treatment with ROSC (6 h), the drug reduced the site-specific phosphorylation and total level of CDK2 and CDK1, as compared to the cells after re-feeding for 10 h. This was accompanied by a slight decrease of the PCNA level. However, the increase of the ratio of S- and later of G_2/M-phase cells upon longer medication with ROSC coincides with the enhancement of activating phosphorylation of CDK2 and CDK1. Total levels of

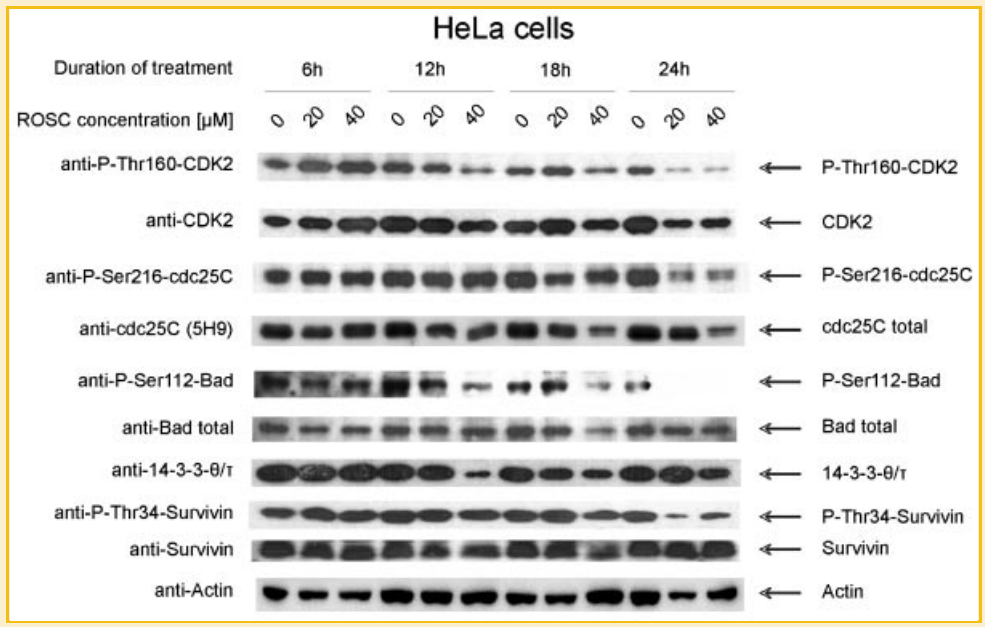

Fig. 5. ROSC-mediated abrogation of phosphorylation of survivin and of Bad in HeLa cells. Untreated control and ROSC-treated HeLa cells were harvested and lysed. Whole cell lysates (WCLs) were loaded on 10% or 12% SDS slab gels. Conditions of immunoblotting as described in detail in Figure 3.

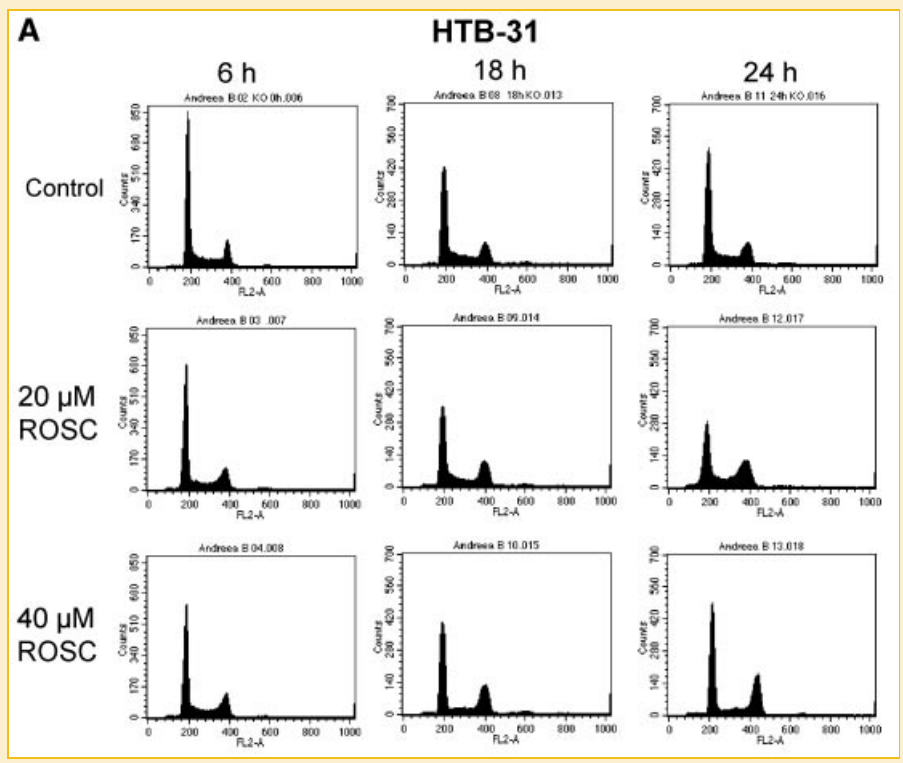

Fig. 6. Effect of ROSC on the cell cycle progression and cell cycle regulators in asynchronously growing HTB-31 cells. A,B: Cell cycle progression. Exponentially growing HTB-31 cells were treated with either 20 or 40 μM ROSC for the indicated periods of time. Control and drug-treated cells were harvested by trypsinization and single cell suspension was used for propidium iodide staining. DNA histograms (A) and diagrams showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G_1 cell population (B). Conditions of staining and measurement as described in detail in the Figure 2. C: ROSC does not activate effector caspase-3 in HTB-31 cells. Untreated and ROSC-treated HTB-31 cells were harvested and lysed. Whole cell lysates (WCLs) were loaded on 12% or 15% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

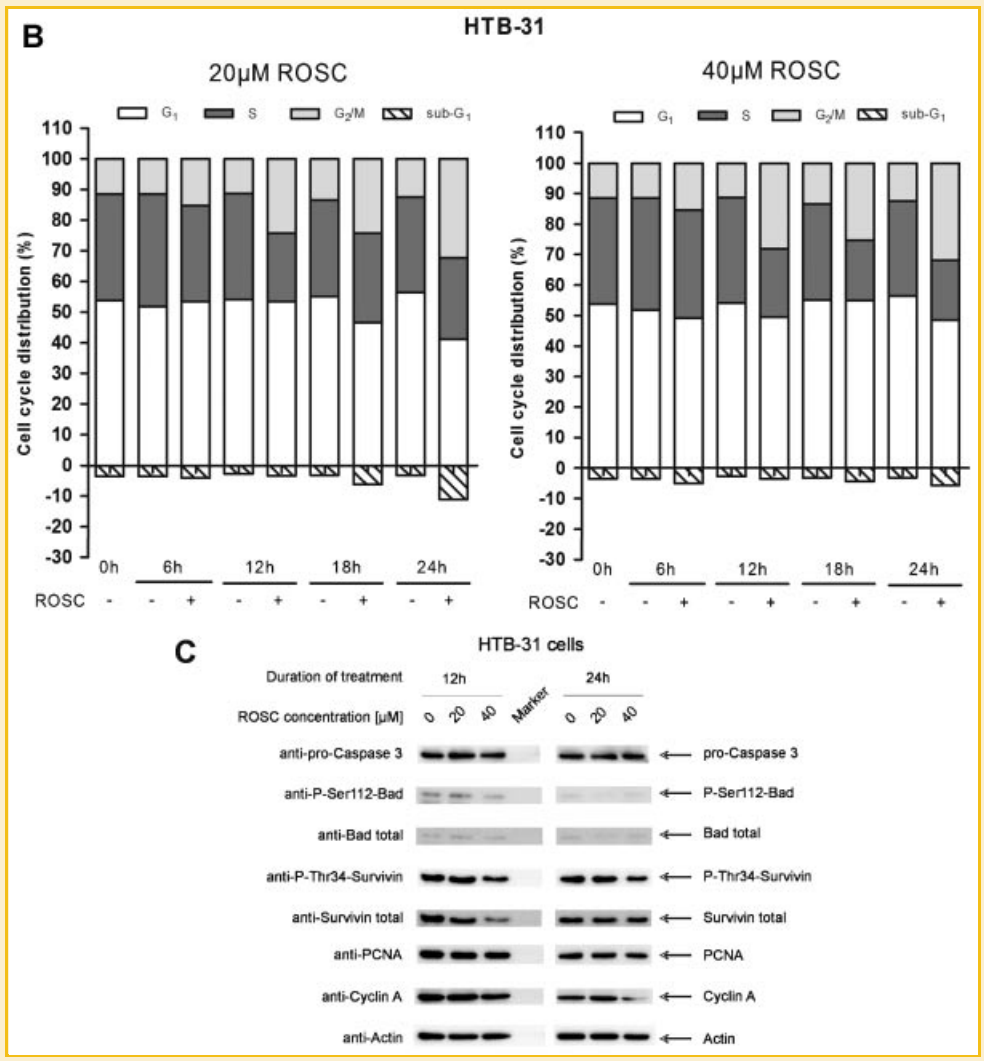

Fig. 6. (*Continued*)

CDK1 and CDK2 also increased (Fig. 9). Although the protein loading was not absolutely equal as evidenced by sequential incubation of the blots with anti-actin antibodies, the fluctuations of the intensity of the CDK1 and CDK2 bands are much stronger than those of actin and thereby support the results gained by flow cytometry.

ROSC MAINTAINS CELL CYCLE BLOCK IN CELLS ARRESTED IN EARLY G_1 PHASE

To verify the inability of ROSC to arrest serum-starved HeLa cells in G_1, we performed the cell cycle analysis of cells synchronized at G_2/M transition followed by a short release from the block and treatment with ROSC. Exposure of HeLa cells for 18 h to NOC at a final concentration of 0.05 μg/ml resulted in a reversible mitotic block. Interestingly, when the cells were released from the block and progressed to early G_1 phase, they became much more susceptible to treatment with ROSC, preventing the cell cycle progression and resulting in a marked G_1 arrest (Fig. 10). The maintenance of G_1 arrest became clearly evident after treatment with ROSC for 12 h; HeLa cells accumulated in the G_1 phase even at lower concentration of ROSC. However, the G_1 arrest was not permanent, the cell cycle progression was rather delayed and after 20 h treatment G_1 population decreased as cells passed through S phase to G_2/M. Progression through S to G_2/M monitored by flow cytometry was also supported by immunoblotting analysis of CDK1, showing not only accumulation of the total CDK1 protein level, but also its phosphorylation at Thr161 (Fig. 11). Similar changes were also observed with the CDK2 level and phosphorylation at Thr160. The DNA profile revealed that after release of HeLa cells from nocodazole-induced mitotic block the ratio of hypoploid cells markedly increased. To check whether after removal of the mitotic blocker DNA is damaged in cells entering the cell cycle, phosphorylation of histone H2A.X at Ser139 was determined. Indeed, after release of HeLa cells from the mitotic block for 4 h the site-specific phosphorylation of H2A.X increased and remained elevated during at least next 20 h in controls as well as ROSC treated cells (Fig. 11). ROSC also increased the cellular levels of p53 protein. However, the p53 increase in cells released from mitotic block was much weaker than that in asynchronously growing cells [Wesierska-Gadek et al., 2008a,b].

DISCUSSION

It is well documented that ROSC, a highly selective CDK inhibitor, influences cell cycle progression as a consequence of interference with multiple CDKs, but different outcomes are observed in different cell lines. Most often, cells arrest at G_2/M transition following ROSC application, as shown for example with asynchronously growing MCF-7 breast carcinoma, HCT-116 colon carcinoma, or K-562 chronic myelogeneous leukaemia cell lines [Penuelas et al., 2003; Wojciechowski et al., 2003; Raynaud et al., 2005], despite weaker inhibition of the mitotic CDK1/cyclin B activity determined in vitro [McClue et al., 2002]. At the same time, however, an increased G_1 population and diminution of the number of S-phase cells was observed in MCF-7 cells or HT-29 colon carcinoma cells [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a, 2006; Krystof et al., 2006], probably as a result of inhibition of CDK2 activity. Other CDK inhibitors were reported to have similar effects on the cell division cycle of animal cells; aminopurvalanol or indirubin-3′-monoxime strongly block G_2/M transition in the Chinese hamster CCL39 lung fibroblast cell line or in SV-40 transformed human HBL-100 breast epithelial cells, respectively [Damiens et al., 2001; Knockaert et al., 2002]. In synchronized cell populations, ROSC, as well as other CDK inhibitors, arrest the cell cycle progression depending on the cell cycle status prior to the onset of the treatment. MCF-7 cells partially synchronized by nocodazole in metaphase were primarily arrested in G_1 phase of the cell cycle after a release from the block and exposure to ROSC [David-Pfeuty, 1999] and the related compound aminopurvalanol induces a G_1 block in the serum-starved CCL39 cell line [Knockaert et al., 2002].

In this work, we studied the anti-proliferative and pro-apoptotic action of ROSC in two human HeLa cervical carcinoma cell lines: HeLa and HTB-31 cells. In HeLa cells the cell cycle is deregulated by infection with HPV-18 virus. Protein products of viral genes E6 and E7 deregulate the host cell growth cycle through binding and inactivating tumor suppressor proteins p53 and pRb, respectively. Considering this fact one might entertain suspicion that inhibition of CDK2 by ROSC would not affect cell cycle progression through G_1/S transition due to inactivation of pRb by E7 oncoprotein, our results were not so straightforward. ROSC indeed reduced the number of viable HeLa cells in the asynchronously growing culture by multiple mechanisms. At a lower dose, it rather directly blocked proliferation, as evidenced by accumulation of cells in G_2/M phases and concomitant decrease of G_1 population. However, at the double ROSC dosage, the frequency of G_1 population increased beginning from 12 h after onset of treatment. This outcome closely coincides with repression of virally encoded E6 [Wesierska-Gadek et al., 2008a] and E7 oncoproteins. The down-regulation of E6 viral product is essential for up-regulation and reactivation of p53 tumor suppressor protein [Wesierska-Gadek et al., 2008a,b], while the repression of E7 protein is crucial for abrogation of HPV-mediated disruption of the cell cycle control. Thus, it is a sign of the reconstitution of the G_1/S checkpoint in HeLa cells. The consequences of the ROSC-mediated effect became clearly evident during the monitoring of the phosphorylation status of pRb protein

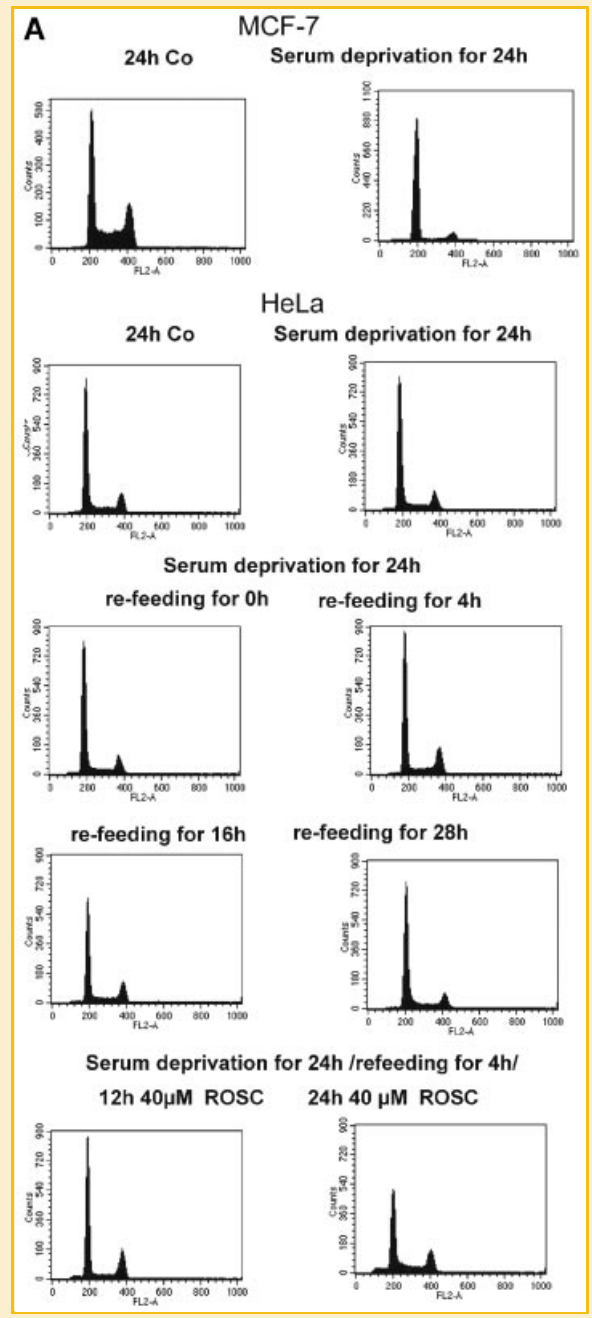

Fig. 7. Lack of G_1 synchronization of HeLa cells after serum deprivation. HeLa cells were plated under normal medium conditions. Twenty four hours after plating, medium was changed and cells were cultivated for indicated periods of time without FCS. At indicated time points cells were refed with serum and cultivated further for indicated periods in the absence or presence of ROSC. Then, cells were collected and prepared for measurement of DNA concentrations as described in more detail in Figure 2. MCF-7 cells were used as a positive control for serum starvation experiment. DNA histograms depicting a representative experiment were prepared using the CellQuest evaluation program (A) and diagrams showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G_1 cell population were constructed for cells kept without ROSC (B) or in the presence of ROSC (C).

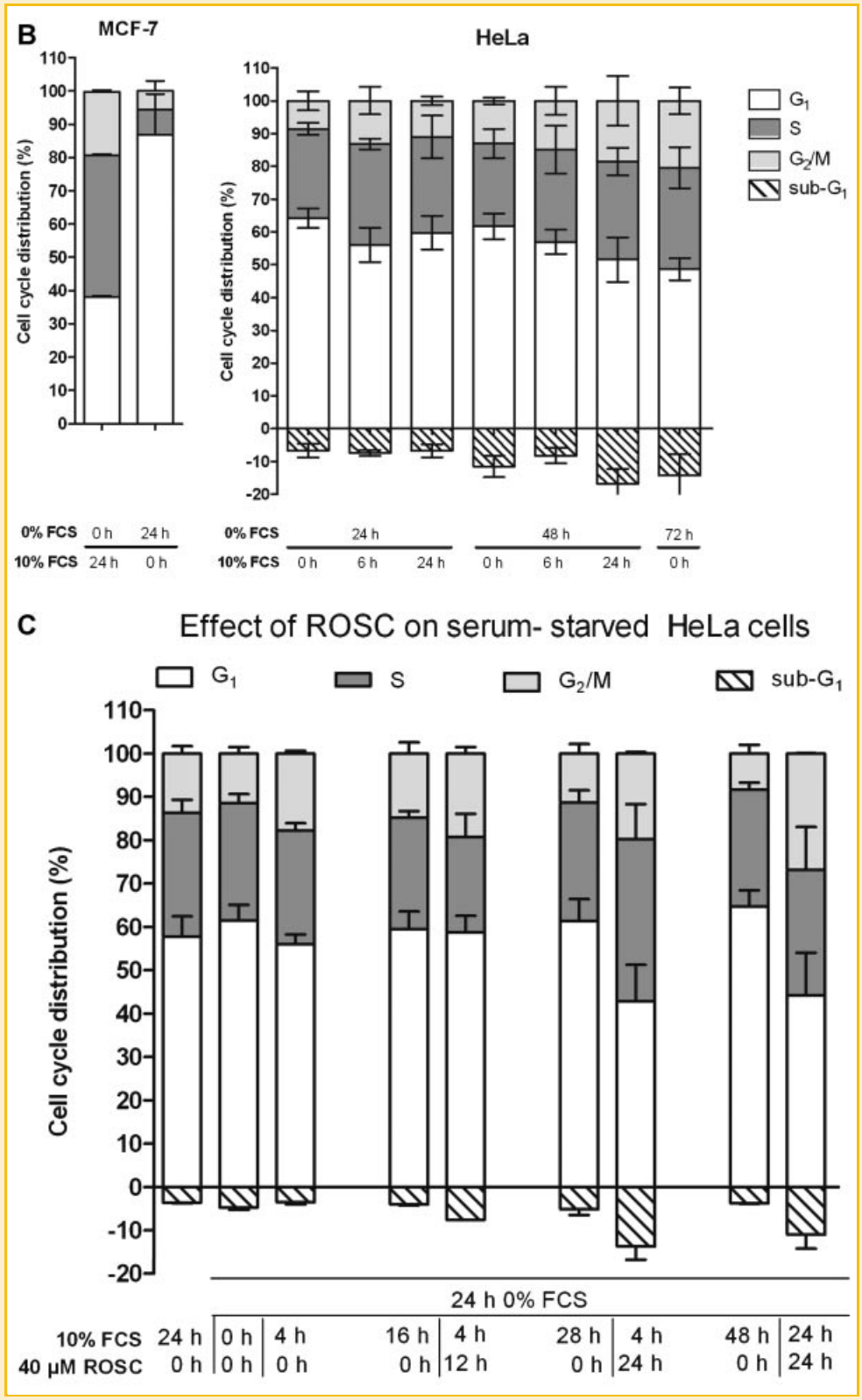

Fig. 7. (*Continued*)

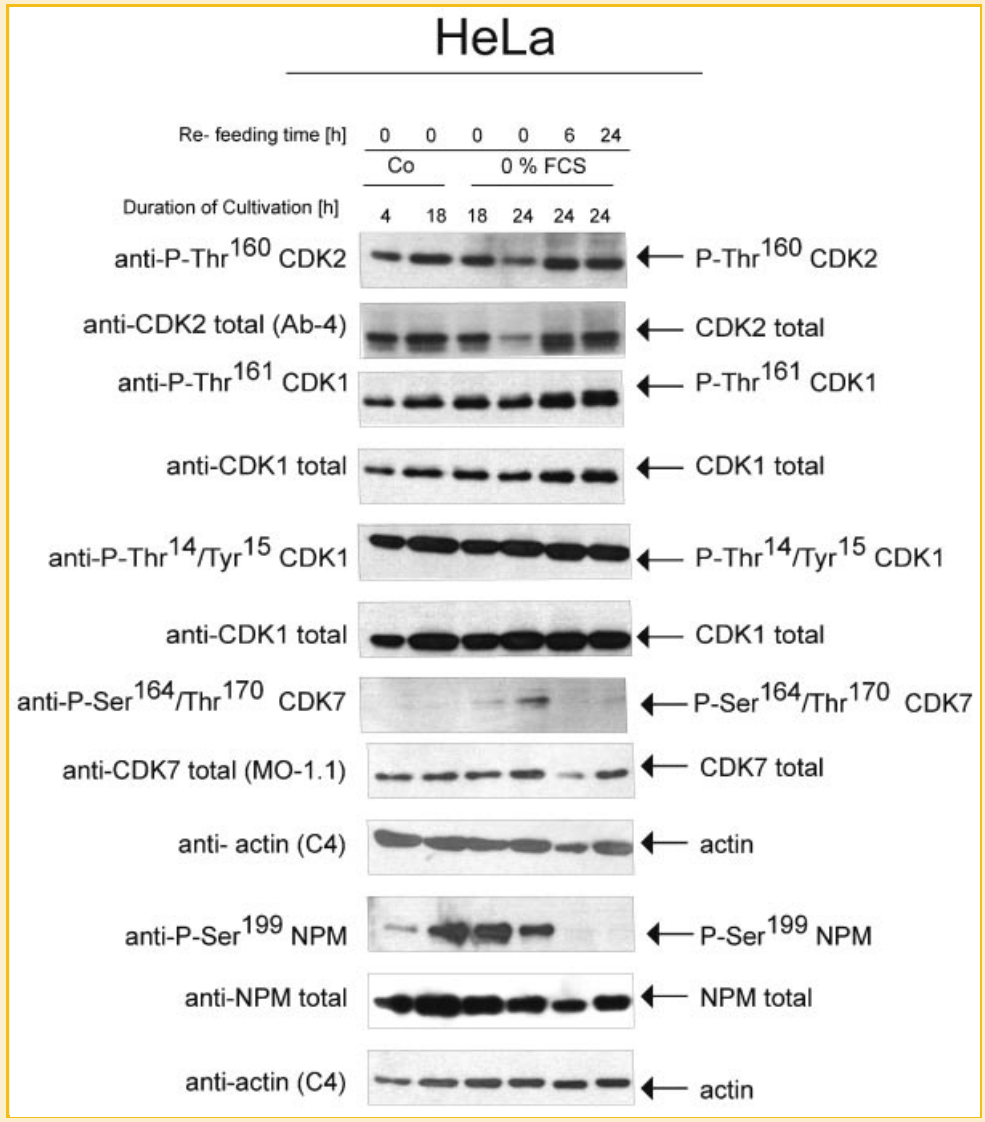

Fig. 8. Cell cycle regulators in HeLa cells after release from serum starvation. Control and serum-starved HeLa cells were harvested at the indicated time points and lysed. Whole cell lysates (WCLs) were loaded on 12% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

that showed decreased signal on immunoblots, as published earlier [Whittaker et al., 2004]. However, pRb is phosphorylated not only by CDK2, but also by CDK4, and therefore we analyzed phosphorylation of nucleophosmin (NPM) at Thr199 as another CDK2-specific substrate. Decreased phosphorylation of NPM following ROSC treatment confirmed inhibition of CDK2 in cells. By phosphorylating this particular residue at NPM, CDK2 allows the centrosome to be duplicated [Matsumoto et al., 1999; Okuda et al., 2000]. Thus, ROSC-mediated dephosphorylation of NPM is another mechanism by which this compound may exert its anti-proliferative action and directly contribute to the G2/M arrest.

ROSC not only limits cell cycle progression of HeLa cells, but after longer period of incubation, also eliminates the cells by induction of apoptosis, like in other cancer cell lines [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a, 2008b]. The ability of ROSC to induce caspase-dependent apoptosis is, however, manifested much more strongly after longer incubation periods and with higher doses of the inhibitor (Fig. 4). It has been shown that inactivation of single cell-cycle regulating CDK leads to cell cycle delay or arrest, while combined genetic or pharmacologic inactivation of CDK1, CDK2, CDK7, and CDK9 induces apoptosis through both E2F- and RNA polymerase II-mediated effects [Cai et al., 2006]. The action of ROSC in HeLa cells probably depends on its relative selectivity towards respective cellular targets, for example, CDK1, CDK2, CDK7, and CDK9 [McClue et al., 2002; Raynaud et al., 2005]. The effect of ROSC on the phosphorylation of CDK7 at Ser164/Thr170 has not been shown yet; it has been only known that ROSC directly inhibits the activity of CDK7 [Hajduch et al., 1999; McClue et al., 2002].

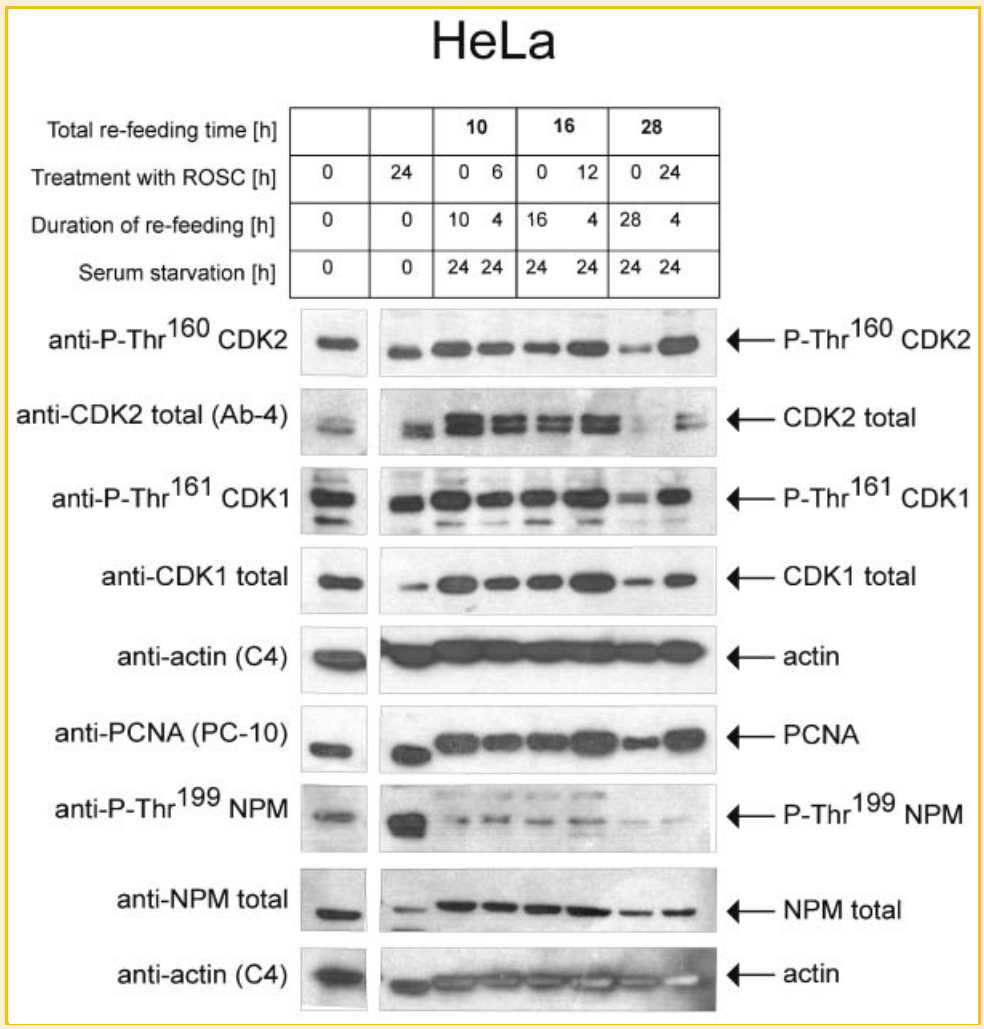

Fig. 9. Effect of ROSC on cell cycle regulators in HeLa cells after release from serum starvation. Untreated control and serum-starved HeLa cells were subsequentially treated with ROSC for indicated periods of time, harvested and lysed. Whole cell lysates (WCLs) were loaded on 12% SDS slab gels. After electrophoretic transfer onto PVDF membrane, the proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by Ponceau S staining and by immunoblotting with anti-actin antibodies.

Similarly to other CDKs, where phosphorylated T-loop uncovers the catalytic site, its dephosphorylation leads to decreased CAK activity [Lolli et al., 2004], which was evidenced here by decreased phosphorylation of CDK1 and CDK2 on their T-loops (Figs. 3A and 8). Although it is not clear yet which kinase is responsible for activation of CDK7 by phosphorylating its T-loop, CDK7 can be activated by its targets CDK1 and CDK2 in vitro [Garrett et al., 2001; Lolli et al., 2004], which are ROSC-sensitive. Hence, activities of all CDK1, CDK2, and CDK7 may decrease in a feedback loop. The situation complicates functional redundancy of multiple CDKs; absence of one CDK is readily compensated with formation of unusual CDK/cyclin complexes as demonstrated by RNAi depletions of CDK1 and CDK2 [Cai et al., 2006]. Moreover, activity of CDK2 is apparently not absolutely dependent on CDK7, as it is able to activate itself by autophosphorylation at Thr160 [Abbas et al., 2007]. By inhibiting CDK1 and CDK2, ROSC in lower doses blocks cell cycle only, while its higher doses, that sufficiently inhibit more kinases in cells (including CDK7), are able to initiate apoptosis, an outcome resembling depletion of multiple CDKs [Cai et al., 2006]. Our results clearly evidence the causal link between activity status of kinases and apoptosis in HeLa cells. We checked the site-specific phosphorylation of some inducers as well as inhibitors of apoptosis. The abrogation of the phosphorylation of two distinct regulators of apoptosis upon treatment with ROSC was achieved solely at higher ROSC dosage and preceded the onset of apoptosis. It is well established that the apoptosis promoting function of Bad protein strongly depends on its phosphorylation status [Harada et al., 1999]. Bad, a cytosolic protein is phosphorylated at serine residues 112 and 136 in response to growth factors resulting in its sequestration by the tau form of 14-3-3 protein. The complex formation with the tau form of 14-3-3 protein abrogates its heterodimerization with Bcl-xL, thereby promoting Bcl-xL-mediated cell survival. ROSC at higher concentration reduced and thereafter abolished Bad phosphorylation already after 12 h. It coincided with the decrease of the total

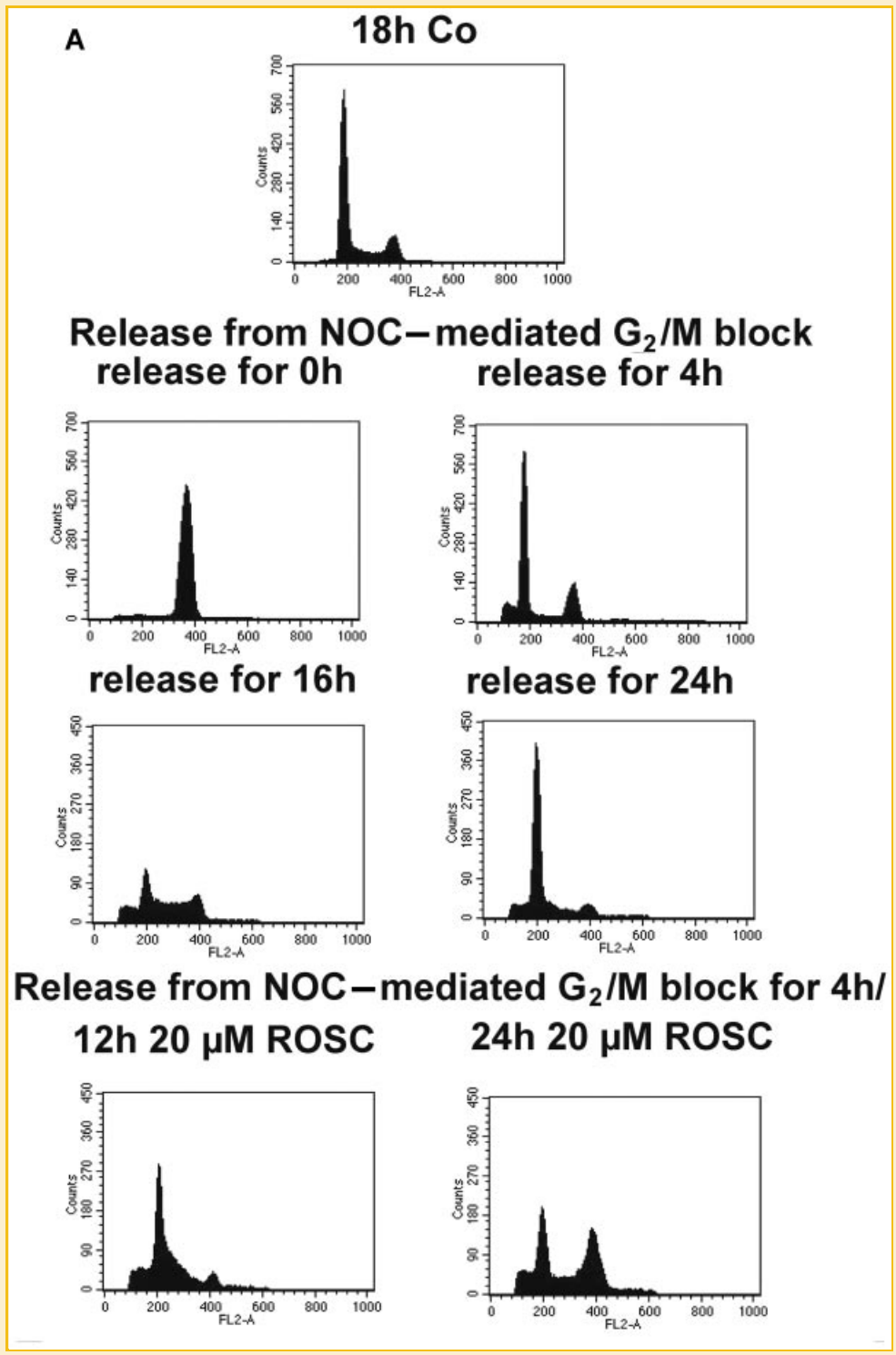

Fig. 10. Effect of ROSC on the cell cycle progression of HeLa cells synchronized by nocodazole. Exponentially growing HeLa cells were synchronized by treatment with nocodazole for 18 h. Then, cells were released from the G_2/M block by medium change and treated for indicated periods of time with either 20 or 40 μM ROSC. Then, cells were collected and prepared for measurement of DNA concentrations as described in more detail in Figure 2. DNA histograms depicting a representative experiment were prepared using the CellQuest evaluation program (A) and diagrams showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G_1 cell population were constructed (B).

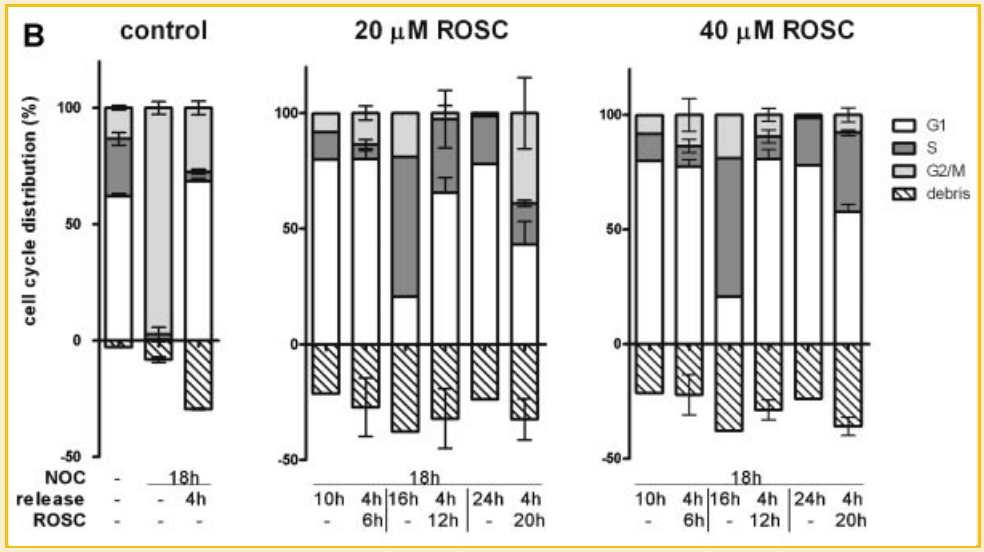

Fig. 10. (*Continued*)

level of 14-3-3 tau protein. ROSC also abrogated the phosphorylation of survivin, the smallest member of the family of inhibitors of apoptois (IAPs). Survivin, a structurally unique IAP lacking a carboxy-terminal RING finger, is a bifunctional protein that acts not only as a suppressor of apoptosis, but is also an essential regulator of cell division [Altieri, 2003]. The highly increased expression of survivin in the majority of human malignancies, resulting in evasion of apoptosis and aberrant regulation of cell division was reported. Remarkably, survivin can be negatively regulated by functional p53 protein [Wesierska-Gadek and Schmid, 2007b] implicating that its expression and activity in response to ROSC treatment may be modulated by two independent mechanisms. The functional status and activity of survivin is regulated by phosphorylation [O'Connor et al., 2000]. Although survivin harbors a few phosphorylation sites that are known to be covalently modified by different cellular kinases, the phosphorylation of Thr34 catalyzed by CDK1 seems to be crucial for its anti-apoptotic activity [Wall et al., 2003]. Threonine 34 is ideally positioned in the acidic knuckle of the survivin molecule [Verdecia et al., 2000] to modulate its interaction with constituents of the apoptotic pathway such as caspase-9 or SMAC/DIABLO and to regulate the binding of client proteins controlling survivin stability. Phosphorylation of survivin at Thr34 increasing protein stability simultaneously enhances its interaction with caspase-9 and SMAC/DIABLO [Song et al., 2003]. Recently, multiple evidences were collected demonstrating that inhibition of survivin phosphorylation on Thr34 [Wall et al., 2003] or expression of non-phosphorylatable survivin mutant $Thr^{34} \rightarrow Ala$ reduce its stability [Blanc-Brude et al., 2003]. It has been also reported that a single amino acid change within amino-terminus converts survivin activity from antiapoptotic to proapoptotic [Song et al., 2004]. These observations encouraged some oncologists to develop new therapeutic approaches based on the prevention of the survivin phosphorylation on Thr34 or its repression [Altieri, 2003; Lu et al., 2004; Zaffaroni et al. 2005].

The observed anti-proliferative and especially pro-apoptotic effect of ROSC is probably enhanced in a p53-dependent manner, because this tumor suppressor accumulated upon longer incubation periods in HeLa cells [Wesierska-Gadek et al., 2008b]. The positive effect of ROSC on activation of p53 and induction of p53-dependent apoptosis has been described several times in the MCF-7 breast carcinoma cell line [David-Pfeuty, 1999; Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. Protein p53, as a transcriptional regulator, operates according to its post-translational modifications, especially phosphorylation and acetylation. For example when phosphorylated at Ser46, that is, at the residue important for its pro-apoptotic activity, p53 can transactivate a set of genes inducing apoptosis (e.g., *Bad*, *Puma*, *Noxa*, *p53AIP1*, *Bax*) and simultaneously suppress expression of anti-apoptotic genes (e.g., *survivin*, *bcl-2*) [Oda et al., 2000]. Moreover, p53 obviously induces apoptosis also independently of transcription; upon a stress signal, cytoplasmic p53 translocates to mitochondria, where it directly activates Bax, leading to the membrane depolarization and caspase activation [Chipuk et al., 2004]. Only recently we have found that ROSC is able to accumulate p53 phosphorylated at Ser46 also in HeLa cells, despite HPV infection [Wesierska-Gadek et al., 2008b]. Interestingly, accumulation of p53 in ROSC-treated HeLa cells is attributable to its stabilization by site-specific phosphorylation [Wesierska-Gadek et al., 2008b]. Moreover, the repression of cellular E6 oncoprotein levels following ROSC treatment allows p53 to restore its physiological activity [Wesierska-Gadek et al., 2008a].

As expected, serum starvation had no influence on progression of HeLa cells through G_1, and even ROSC, applied to the cells shortly after the release from starvation, was not able to maintain cells in G_1 phase, probably due to inactivation of the key G_1-regulator pRb by E7 oncoprotein. Although ROSC at high dosage down-regulates E7 oncoprotein, this effect takes place with some delay. Reduction of E7 in asynchronously growing HeLa cells was observed after 18 h. Conversely, populations in S-phase and G_2/M phases were significantly delayed following ROSC treatment for 24 h, probably as a consequence of unphosphorylated NPM that further block onset of mitosis [Matsumoto et al., 1999; Okuda et al., 2000]. In fact, the outcome is very similar to that in asynchronously growing cells,

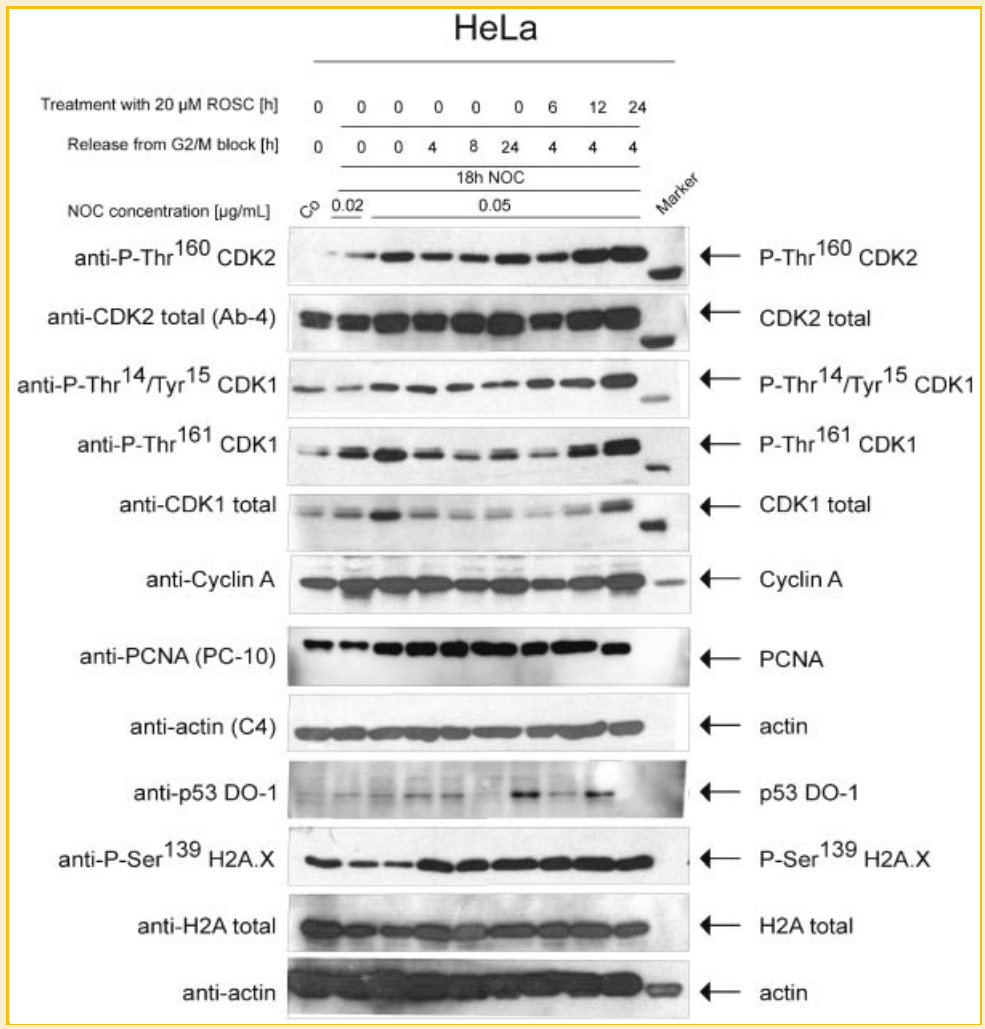

Fig. 11. Effect of ROSC on cell cycle regulators in NOC-synchronized HeLa cells. Unsynchronized control and NOC-synchronized HeLa cells were treated with ROSC for indicated periods of time, harvested and lysed. Whole cell lysates (WCLs) were loaded on 12 or 10% SDS slab gels. After electrophoretic transfer onto PVDF membrane, protein loading and transfer was checked by Ponceau S staining. The proteins as well as their phosphorylation statuses were examined by incubation with specific antibodies. The equal protein loading was checked by immunoblotting with anti-actin antibodies.

reflecting the heterogeneity of HeLa cells cultivated without the serum.

Although ROSC alone was not able to arrest HeLa cells in G_1 phase in either asynchronous or serum-starved culture, when released from nocodazole arrest, HeLa cells markedly delayed progression through G_1 phase when subsequently treated with ROSC (Fig. 10B). As the cells further proliferated and passed slowly through S phase to G_2, they also accumulated Thr-phosphorylated forms of CDK1 and CDK2 (Fig. 11).

In summary, ROSC has been shown to have different effects on HeLa cells depending on the concentration used and on the cell cycle status prior to the onset of treatment. Generally, lower doses reduce the proliferation rate by inhibition of cyclin-dependent kinases, while higher doses induce caspase-dependent apoptosis in HeLa cells. Moreover, the results also show that inhibition of CDKs by ROSC in cells lacking the G_1/S restriction checkpoint has different outcomes depending on the cell cycle status of the treated cells. Considering the fact that after radiotherapy cancer cells are blocked at G_2/M, the ability of ROSC to induce apoptosis in G_2/M arrested cells may be of therapeutic importance.

ACKNOWLEDGMENTS

The authors thank Dr. I. Herbacek for performing flow cytometric measurements. This work was supported by a grant from the Austrian Science Fund FWF (P-19894-B11).

REFERENCES

Abbas T, Jha S, Sherman NE, Dutta A. 2007. Autocatalytic phosphorylation of CDK2 at the activating Thr160. Cell Cycle 6:843–852.

Alessi F, Quarta S, Savio M, Riva F, Rossi L, Stivala LA, Scovassi AI, Meijer L, Prosperi E. 1998. The cyclin-dependent kinase inhibitors olomoucine and

roscovitine arrest human fibroblasts in G1 phase by specific inhibition of CDK2 activity. Exp Cell Res 245:8–18.

Altieri DC. 2003. Validating survivin as a cancer therapeutic target. Nat Rev 3:46–54.

Balsitis S, Dick F, Dyson N, Lambert PF. 2006. Critical roles for non-pRb targets of human papillomavirus type 16 E7 in cervical carcinogenesis. Cancer Res 66:9393–9400.

Besson A, Dowdy SF, Roberts JM. 2008. CDK inhibitors: Cell cycle regulators and beyond. Dev Cell 14:159–169.

Blanc-Brude OP, Mesri M, Wall NR, Plescia J, Dohi T, Altieri DC. 2003. Therapeutic targeting of the survivin pathway in cancer: Initiation of mitochondrial apoptosis and suppression of tumor-associated angiogenesis. Clin Cancer Res 9:2683–2692.

Cai D, Byth KF, Shapiro GI. 2006. AZ703, an imidazo[1,2-a]pyridine inhibitor of cyclin-dependent kinases 1 and 2, induces E2F-1-dependent apoptosis enhanced by depletion of cyclin-dependent kinase 9. Cancer Res 66:435–444.

Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M, Green DR. 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303:1010–1014.

Dai Y, Grant S. 2003. Cyclin-dependent kinase inhibitors. Curr Opin Pharmacol 4:362–370.

Damiens E, Baratte B, Marie D, Eisenbrand G, Meijer L. 2001. Anti-mitotic properties of indirubin-3′-monoxime, a CDK/GSK-3 inhibitor: Induction of endoreplication following prophase arrest. Oncogene 20:3786–3797.

David-Pfeuty T. 1999. Potent inhibitors of cyclin-dependent kinase 2 induce nuclear accumulation of wild-type p53 and nucleolar fragmentation in human untransformed and tumor-derived cells. Oncogene 18:7409–7422.

Fischer PM, Lane DP. 2000. Inhibitors of cyclin-dependent kinases as anticancer therapeutics. Curr Med Chem 12:1213–1245.

Fisher RP. 2005. Secrets of a double agent: CDK7 in cell-cycle control and transcription. J Cell Sci 118:5171–5180.

Fry DW, Harvey PJ, Keller PR, Elliott WL, Meade M, Trachet E, Albassam M, Zheng X, Leopold WR, Pryer NK, Toogood PL. 2004. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. Mol Cancer Ther 3:1427–1438.

Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA. 1997. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. Genes Dev 11:2090–2100.

Garrett S, Barton WA, Knights R, Jin P, Morgan DO, Fisher RP. 2001. Reciprocal activation by cyclin-dependent kinases 2 and 7 is directed by substrate specificity determinants outside the T loop. Mol Cell Biol 21:88–99.

Hajduch M, Havlicek L, Vesely J, Novotny R, Mihál V, Strnad M. 1999. Synthetic cyclin dependent kinase inhibitors. New generation of potent anticancer drugs. Adv Exp Med Biol 457:341–353.

Harada H, Becknell B, Wilm M, Mann M, Huang LJ, Taylor SS, Scott JD, Korsmeyer SJ. 1999. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol Cell 3:413–422.

Havlicek L, Hanus J, Vesely J, Leclerc S, Meijer L, Shaw G, Strnad M. 1997. Cytokinin-derived cyclin-dependent kinase inhibitors: Synthesis and cdc2 inhibitory activity of olomoucine and related compounds. J Med Chem 40(4): 408–412.

Helt AM, Galloway DA. 2003. Mechanisms by which DNA tumor virus oncoproteins target the Rb family of pocket proteins. Carcinogenesis 24:159–169.

Hume AJ, Finkel JS, Kamil JP, Coen DM, Culbertson MR, Kalejta RF. 2008. Phosphorylation of retinoblastoma protein by viral protein with cyclin-dependent kinase function. Science 320:797–799.

Knockaert M, Lenormand P, Gray N, Schultz P, Pouysségur J, Meijer L. 2002. p42/p44 MAPKs are intracellular targets of the CDK inhibitor purvalanol. Oncogene 21:6413–6424.

Krystof V, Cankar P, Frysová I, Slouka J, Kontopidis G, Dzubák P, Hajdúch M, Srovnal J, de Azevedo WF, Jr., Orság M, Paprskárová M, Rolcík J, Látr A, Fischer PM, Strnad M. 2006. 4-arylazo-3,5-diamino-1H-pyrazole CDK inhibitors: SAR study, crystal structure in complex with CDK2, selectivity, and cellular effects. J Med Chem 49:6500–6509.

Lolli G, Lowe ED, Brown NR, Johnson LN. 2004. The crystal structure of human CDK7 and its protein recognition properties. Structure 12:2067–2079.

Lu B, Mu Y, Cao C, Zeng F, Schneider S, Tan J, Price J, Chen J, Freeman M, Hallahan DE. 2004. Survivin as a therapeutic target for radiation sensitization in lung cancer. Cancer Res 64:2840–2845.

Malumbres M, Barbacid M. 2005. Mammalian cyclin-dependent kinases. Trends Biochem Sci 30:630–641.

Malumbres M, Barbacid M. 2007. Cell cycle kinases in cancer. Curr Opin Genet Dev 17:60–65.

Malumbres M, Pevarello P, Barbacid M, Bischoff JR. 2008. CDK inhibitors in cancer therapy: What is next? Trends Pharmacol Sci 29:16–21.

Matsumoto Y, Hayashi K, Nishida E. 1999. Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. Curr Biol 9:429–432.

McClue SJ, Blake D, Clarke R, Cowan A, Cummings L, Fischer PM, MacKenzie M, Melville J, Stewart K, Wang S, Zhelev N, Zheleva D, Lane DP. 2002. In vitro and in vivo antitumor properties of the cyclin dependent kinase inhibitor CYC202 (R-roscovitine). Int J Cancer 102:463–468.

McLaughlin-Drubin ME, Huh KW, Münger K. 2008. The human papillomavirus type 16 E7 oncoprotein associates with E2F6. J Virol DOI: 10.1016/j.virol.2008.10.006.

Nguyen DX, Westbrook TF, McCance DJ. 2002. Human papillomavirus type 16 E7 maintains elevated levels of the cdc25A tyrosine phosphatase during deregulation of cell cycle arrest. J Virol 76:619–632.

O'Connor DS, Grossman D, Plescia J, Li FZ, Zhang H, Tognin S, Marchisio PC, Altieri DC. 2000. Regulation of apoptosis at cell division by $p34^{cdc2}$ phosphorylation of survivin. Proc Natt Acad Sci USA 97:13103–13107.

Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y, Taya Y. 2000. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 102:849–862.

Okuda M, Horn HF, Tarapore P, Tokuyama Y, Smulian AG, Chan PK, Knudsen ES, Hofmann IA, Snyder JD, Bove KE, Fukasawa K. 2000. Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell 103:127–140.

Penuelas S, Alemany C, Noe V, Ciudad CJ. 2003. The expression of retinoblastoma and Sp1 is increased by low concentrations of cyclin-dependent kinase inhibitors. Eur J Biochem 270:4809–4822.

Raynaud FI, Whittaker SR, Fischer PM, McClue S, Walton MI, Barrie SE, Garrett MD, Rogers P, Clarke SJ, Kelland LR, Valenti M, Brunton L, Eccles S, Lane DP, Workman P. 2005. In vitro and in vivo pharmacokinetic-pharmacodynamic relationships for the trisubstituted aminopurine cyclin-dependent kinase inhibitors olomoucine, bohemine and CYC202. Clin Cancer Res 11:4875–4887.

Schang LM, Coccaro E, Lacasse JJ. 2005. Cdk inhibitory nucleoside analogs prevent transcription from viral genomes. Nucleos Nucleot Nucl Acid 24:829–837.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63:1129–1136.

Shapiro GI. 2006. Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol 24:1770–1783.

Song Z, Yao X, Wu M. 2003. Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. J Biol Chem 278:23130–23140.

Song Z, Liu S, He H, Hoti N, Wang Y, Feng S, Wu M. 2004. A single amino acid change (Asp53-Ala53) converts survivin from antiapoptotic to proapoptotic. Mol Biol Cell 15:1287–1296.

Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, Noel JP. 2000. Structure of the human anti-apoptotic protein survivin reveals a dimeric rearrangement. Nat Struct Biol 7:602–608.

Vindelov LL, Christensen IJ, Nissen NI. 1983. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3:323–327.

Wall NR, O'Connor DS, Plescia J, Pommier Y, Altieri DC. 2003. Suppression of survivin phosphorylation on Thr34 by flavopiridol enhances tumor cell apoptosis. Cancer Res 63:230–235.

Wesierska-Gadek J, Schmid G. 2000. Overexpressed poly(ADP-ribose) polymerase delays the release of rat cells from p53-mediated G(1) checkpoint. J Cell Biochem 80:85–103.

Wesierska-Gadek J, Schmid G. 2007b. Transcriptional repression of anti-apoptotic proteins mediated by the tumor suppressor protein p53. Cancer Ther 5:203–212.

Wesierska-Gadek J, Schloffer D, Kotala V, Horky M. 2002. Escape of p53 protein from E6-mediated degradation in HeLa cells after cisplatin therapy. Int J Cancer 101:128–136.

Wesierska-Gadek J, Gueorguieva M, Horky M. 2005a. Roscovitine-induced up-regulation of p53AIP1 protein precedes the onset of apoptosis in human MCF-7 breast cancer cells. Mol Cancer Ther 4:113–124.

Wesierska-Gadek J, Gueorguieva M, Ranftler C, Zerza-Schnitzhofer G. 2005b. A new multiplex assay allowing simultaneous detection of the inhibition of cell proliferation and induction of cell death. J Cell Biochem 96:1–7.

Wesierska-Gadek J, Schreiner T, Gueorguieva M, Ranftler C. 2006. Phenol red reduces ROSC mediated cell cycle arrest and apoptosis in human MCF-7 cells. J Cell Biochem 98:1367–1379.

Wesierska-Gadek J, Gueorguieva M, Kramer MP, Ranftler C, Sarg B, Lindner H. 2007a. A new, unexpected action of olomoucine, a CDK inhibitor, on normal human cells: Up-regulation of CLIMP-63, a cytoskeleton-linking membrane protein. J Cell Biochem 102:1405–1419.

Wesierska-Gadek J, Schmitz ML, Ranftler C. 2007c. Roscovitine-activated HIP2 kinase induces phosphorylation of wt p53 ar Ser-46 in human MCF-7 breast cancer cells. J Cell Biochem 100:865–874.

Wesierska-Gadek J, Hajek SB, Sarg B, Wandl S, Walzi E, Lindner H. 2008a. Pleiotropic effects of selective CDK inhibitors on human normal and cancer cells. Biochem Pharmacol 76:1503–1514.

Wesierska-Gadek J, Wandl S, Kramer M, Pickem C, Krystof V, Hajek SB. 2008b. Roscovitine up-regulates p53 protein and induces apoptosis in human HeLaS3 cervix carcinoma cells. J Cell Biochem 105:1161–1171.

Whittaker SR, Walton MI, Garrett MD, Workman P. 2004. The cyclin-dependent kinase inhibitor CYC202 (R-roscovitine) inhibits retinoblastoma protein phosphorylation, causes loss of Cyclin D1, and activates the mitogen-activated protein kinase pathway. Cancer Res 64:262–272.

Wojciechowski J, Horky M, Gueorguieva M, Wesierska-Gadek J. 2003. Rapid onset of nucleolar disintegration preceding cell cycle arrest in roscovitine-induced apoptosis of human MCF-7 breast cancer cells. Int J Cancer 106:486–495.

Zaffaroni N, Pennati M, Daidone MP. 2005. Survivin as a target for new anticancer intervention. J Cell Mol Med 9:360–372.